Express Mailing Label No. EV138525133US
Date of Deposit: July 16, 2003

ANTI-INFLAMMATORY FORMULATIONS

This application is a continuation in part of U.S.S.N. 10/345,856, filed on January 16, 2003 which claims the benefit of provisional application U.S.S.N. 60/350,298, filed on January 16, 2002, the entire contents of which is hereby incorporated by reference in their entireties.

BACKGROUND OF THE INVENTION

The invention relates to control of ocular inflammation.

Aqueous tear-deficient dry eye syndrome is a disruption of the ocular surface-lacrimal gland homeostatic cycle. It is characterized by dry inflammation of the lacrimal gland, and presence of a dense infiltrate of inflammatory cells in and around the tear duct causing high localized expression of pro-inflammatory cytokines. A particularly debilitating form of the disorder is dry, age-related macular degeneration which affects a substantial fraction of the population older than 65 years and is currently incurable

SUMMARY OF THE INVENTION

The invention features an anti-inflammatory composition which is associated with reduced adverse side effects compared to conventional anti-inflammatory drugs. Additionally, the combination of the individual components of the composition results in a greater anti-inflammatory effect than the anti-inflammatory effect of the individual components when administered singularly. The anti-inflammatory compositions are useful in protecting ocular tissue from inflammation related and oxidative damage.

The anti-inflammatory composition contains a carotinoid and a polyphenol. The carotenoid is mixed carotenoid compound, an astaxanthin or a zeaxanthin. The polyphenol is curcuma longa root powder, green tea, grape seed extract, or a citrus bioflavonoid. Alternatively, the polyphenol is a cox-2 inhibitor such as a quercetin, a bilberry extract, a hops PE, blueberry powder or tart cherry powder.

In some embodiments the anti-inflammatory composition contains a glutathione precursor, a vitamin anti-oxidant or an alpha lipoic acid. The composition optionally also contains a trace mineral. The glutathione precursor is taurine or N-acetyl-L-cysteine. A vitamin anti-oxidant includes for example, Vitamin A, Vitamin B, Vitamin C, or Vitamin E, or Vitamin D.

The invention also includes a method of reducing inflammation in an ocular tissue Inflammation is inhibited by administering to an inflamed tissue an anti-inflammatory composition described above. An inflamed tissue is characterized by redness, pain and swelling of the tissue. The tissue includes ocular tissue. For example, the ocular tissue is sclera tissue, iris tissue, cornea tissue, pupil tissue, lens tissue, conjuctiva tissue, vitreous tissue, choroids tissue, macula tissue or retina tissue. Optionally, the tissue is contacted with an omega-3 fatty acid such as eicosapentaenoic acid or docosahexaenoic acid.

Also within the invention is a method of alleviating a symptom of an ocular inflammatory disease such as dry eye or macular degeneration by administering to a subject one or more of the anti-inflammatory compositions described above. The subject is a mammal, such as human, a primate, mouse, rat, dog, cat, cow, horse, pig. The subject is suffering from or at risk of developing an ocular inflammatory disease. A subject suffering from or at risk of developing inflammatory is identified by methods known in the art, e.g., itching, burning irritation, redness, blurred vision, or difficulty reading. Symptoms of inflammation include pain, redness and swelling of the affected tissue.

The composition is administered systemically. Alternatively, the composition is administered locally. For example, the composition is administered by directly contacting an inflamed ocular tissue with the composition. The compositions are administered prior to after development of ocular inflammation as a prophylaxis; or after development of ocular inflammation as a therapeutic. Optionally, the subject is co-administered a composition containing omega-3 fatty acids.

The invention features further features an anti-inflammatory composition, which is associated with reduced adverse side effects such as decreased cell-mediated immunity compared to conventional anti-inflammatory drugs. The anti-inflammatory composition

contains a lipid-soluble antioxidant carotenoid. In some embodiments, the composition does not contain a beta-carotene compound. The composition may also contain a water-soluble antioxidant (vitamin C or ascorbic acid) and/or a ginkgolide.

Accordingly, the invention provides a composition containing a lipid soluble antioxidant and a water soluble antioxidant. The lipid soluble antioxidant is a carotenoid compound. The carotenoid compound is astaxanthin or an ester thereof or a vitamin such as ascorbic acid. Alternatively, the water soluble antioxidant is a ginkgolide such as a terpene trilactone selected from the group consisting of Gingkolide A, Gingkolide B, Gingkolide C, Gingkolide J, Gingkolide M, and bilobalide. The gingkolide composition preferably exhibits one or more of the following activities: (i) platelet activating factor receptor (PAFR) antagonist activity; (ii) PLA2-inhibitory capability; (iii) COX-2-inhibitory capability; and (iv) the capability to inhibit cAMP phosphodiesterase. Preferably, the composition exhibits all of the aforementioned activities. For example, the ginkgolide composition contains Egb 761. The composition optionally also contains an histamine release inhibitor such as a cetirizine compound and/or an azelastine compound. In preferred embodiments, the composition contains a mixture of an asaxanthin compound, a ginkgolide compound, and an ascorbic acid compound.

The invention also includes a method of suppressing inflammation in a mammal. The method is carried out by co-administering of astaxanthin or a derivative thereof, vitamin C, and one or more classes of gingkolide in such amounts so as to provide an additive or synergistic anti-inflammatory effect. Preferably, the gingkolide is administered at a dose that preferentially inhibits expression of an inflammatory cytokine much as IL-8, IL-1α, IL-1β, TNF-α or IL-6.

For example, the invention provides a method of inhibiting activation of an immune cell by contacting the immune cell (e.g., a T cell or a mast cell) with the composition(s) described above. Also within the invention is a method of alleviating a symptom of an inflammatory disease by administering to a mammal suffering from or at risk of developing the disease one or more of the anti-inflammatory composition described above. In one example, the composition is administered systemically. Alternatively, the composition is administered locally. For example, the composition is

administered by directly contacting an inflammed tissue with the composition. The tissue to be directly contacted is dermal tissue in the case of skin inflammatory diseases such as psoriasis. For asthma, the tissue is pulmonary tissue, e.g., bronchoalveolar tissue. In the former case, the compositions are administered topically, e.g., by contacting skin with a cream, lotion, or ointment. In the latter case, pulmonary tissue is contacted by inhaling a composition, e.g., a liquid or powder aspirate containing the mixture of anti-inflammatory compounds.

Antioxidants such as carotenoids are co-administered with other agents to reduce inflammation. For example, astaxanthin (or esters thereof), vitamin C, and the gingkolide(s) are administered simultaneously or consecutively. For example, the gingkolide(s) is first administered followed by astaxanthin, followed by vitamin C. Alternatively, astaxanthin is administered first and then the gingkolide(s) and then vitamin C. In another regimen, vitamin C is administered first, followed by astaxanthin, followed by the ginkgolide(s); or vitamin C is administered following administration of either astaxanthin or the ginkgolide, followed by administration of the third component. The combination of compounds is administered in the presence or absence of a traditional anti-inflammatory agent such as a corticosteroid or non-steroidal anti-inflammatory agent.

Such a co-administration regimen is useful to inhibit inflammation in a mammal. For example, each of the aforementioned thre classes of compounds are administered prior to after development of inflammation as a prophylaxis; or after development of inflammation as a therapeutic.

The antioxidant and gingkolide compounds described are also useful in combination with nonsteroidal anti-inflammatory drugs (NSAIDs) to reduce the dose of NSAID required to achieve a desired clinical effect such as reduction of symptoms associated with Alzheimer's Disease. Combined with histamine release blockers such as cetirizine, the antioxidant and gingkolide compounds augment the clinical effect (e.g., reduction of allergy symptoms such as itching) of the histamine release blocker, thereby permitting administration of a lower dose of the histamine release blocker. Coadminstraion of an antioxidant and/or a gingkolide compound reduces adverse side effects associated with many known anti-inflammatory and anti-allergy medications.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a bar graph showing the effect of astaxanthin (ASX) on immune activation of human PBMC. Cells cultured 24h at 37°C, 5% CO_2 in RPMI 1640, 10% FCS with 50 mg/ml PHA and ASX were evaluated by 3-color flow cytometry for immune activation as %CD3+ cells induced to express membrane-bound CD25 (IL-2 receptor). Stimulation indices (SI) were determined as the ratio of %CD3+CD25+ cells in fully-stimulated cultures treated with PHA alone, to those cultured with PHA plus ASX. Results are representative of independent assays conducted on cells of 6 - 8 asthmatic donors participating in this study. Significance in comparison with fully-stimulated cultures: (*: $p<0.05$)

Fig. 2 is a bar graph showing the effect of ginkgolide B (GB) on immune activation of human PBMC. Cells cultured 24h at 37°C, 5% CO_2 in RPMI 1640, 10% FCS with 50 mg/ml PHA and GB were evaluated by 3-color flow cytometry for immune activation as %CD3+ cells induced to express membrane-bound CD25 (IL-2 receptor). Stimulation indices (SI) were determined as the ratio of %CD3+CD25+ cells in fully-stimulated cultures treated with PHA alone, to those cultured with PHA plus GB. Results are representative of independent assays conducted on cells of 6 - 7 asthmatic donors participating in this study. Significance in comparison with fully-stimulated cultures: (*: $p<0.05$)

Fig. 3 is a bar graph showing the effect of astaxanthin (ASX) plus ginkgolide B (GB) on immune activation of human PBMC. Cells cultured 24h with 50 mg/ml PHA and selected combinations of ASX + GB were evaluated by 3-color flow cytometry for immune activation as %CD3+ cells induced to express CD25 (IL-2 receptor). Stimulation indices (SI) are determined as the ratio of %CD3+CD25+ cells in fully-stimulated cultures treated with PHA alone, to those cultured with PHA plus selected combinations of ASX + GB. Results are representative of independent assays conducted

on cells of 4 - 7 healthy adult donors participating in this study. Significance in comparison with fully-stimulated cultures: (*: $p<0.05$)

Figs 4A-4B are bar graphs showing the effect of cetirizine (Zyrtec/CTZ) versus azalestene (AZE) on immune activation of human PBMC. Cells cultured 24h at 37°C, 5% CO_2 in RPMI 1640, 10% FCS with 50 mg/ml PHA and either CTZ (4A) or AZE (Fig. 4B) are evaluate by 3-color flow cytometry for immune activation as % CD3+ cells induced to express membrane-bound CD25 (IL-2 receptor). Results are representative of independent assays conducted on cells of 7 - 12 asthmatic donors. Significance (P) in comparison with fully-stimulated culture: (*:$p<0.05$)

DETAILED DESCRIPTION

The compositions described herein are useful to prevent inflammation, and improve the clinical prognosis for patients suffering from inflammatory disease. The combined action of a lipid-soluble carotenoid (principally astaxanthin) with vitamin C and one or more components of a *Ginkgo biloba* extract mediates prevention or suppression of disease-associated inflammation.

Astaxanthin

Astaxanthin (3,3'-dihydroxy-4,4'-diketo-ß-carotene) is a carotenoid produced by several natural sources, including: the marine algae *Haematococcus pluvialis*; and the pink yeast *Xanthophyllomyces dendrorhous*. It is obtained directly from either aforementioned organism; or alternatively by extraction from by-products of crustacea such as the Antarctic krill *Euphausia superba*. Its molecular structure is similar to that of carotenoid beta-carotene, however small differences in structure confer large differences in the chemical and biological properties of the two molecules. In particular, astaxanthin is superior to beta-carotene in its capacity to scavenge free radicals. It exhibits strong antioxidant properties and confers protection against lipid peroxidation and oxidative damage of LDL-cholesterol, cell membranes, cells, and tissues. Beneficial effects mediated by astaxanthin in mammals are known to include: increased boar semen volume and piglet litter size and survival rate when fed to pigs; augmentation of anti-

stress agents administered to farm animals and household pets; improved immunity; and suppression of tumor growth.

Additionally, esterified astaxanthin from *Haematococcus pluvialis* algal meal is therapeutic for muscular dysfunction such as exertional rhabdomyolysis (also known as exertional myopathy, tying-up syndrome, azoturia, or Monday morning sickness) in horses; and for gastrointestinal tract inflammation due to infections by *Helicobacter* sp. bacteria.

Gingkolides

Ginkgo Biloba. is a plant, the leaves, roots, and fruit of which have been used for medicinal purposes for centuries. Extracts of various parts of the plant are commercially available. A gingkolide, or *Ginkgo biloba* extract contains one or more biologically active components such as an antioxidant component and an PAFR antagonist component. For example, an extract is made from ginkgo leaves and used at a concentration that contains about 24 - 25% ginkgo-flavone-glycosides. The extract may also contain terpenoids such as Egb761 or LI-1370. For example, the preparation contains 24% ginkgo-flavone glycosides and 6% terpenoids. The ginkgo-flavone glycosides are sometimes referred to as heterosides. EGb761 is a commercially available leaf extract of Ginkgo biloba, containing: GA, GB, GC, GJ, GM and bilobalide.

Naturally-occurring Ginkgo biloba contains: (A) biflavones such as amentoflavone, bilobetin, sequoiaflavone, ginkgetin, isoginkgetin, Sciadopitysin; (B) flavonol glycosides; (C) terpene trilactones, such as Gingkolide A, Gingkolide B, Gingkolide C, Gingkolide J, Gingkolide M and bilobalide; (D) rutin; (E) quercetin; and (F) a 30 kDa Ginkgo biloba glycoprotein, which reacts with antiserum against beta 1→2 xylose-containing N-glycans. Each component or combinations thereof are isolated from crude extracts of the plant using methods known in the art.

Alphabetically-labeled series of ginkgolide derivatives are further characterized as follows. Ginkgolide A (GA) is a leaf extract containsin terpene trilactone. This gingkolide is a PAFR antagonist, but has no apparent antioxidant properties. It is also known as BN52020, CAS 15291-75-5. Ginkgolide B (GB) is a leaf extract containing terpene trilactone. It is a PAFR antagonist, with antioxidant properties and may be

referred to as BN52021 or CAS 15291-77-7. GC, ginkgolide C: a terpene trilactone, leaf extract. A PAFR antagonist, with antioxidant properties. Ginkgolide J (GJ) is a leaf extract containing terpene trilactone with PAFR antagonist activity and antioxidant properties. Ginkgolide M (GM) is a root extract containing terpene trilactone. This gingkolide has PAFR antagonist activity and antioxidant properties. Bilobalide (a sesquiterpene trilactone) is primarily an antioxidant. *Ginkgo biloba* extract (EGb 761) is a clinically safe, nontoxic, and easily-produced product with a wide range of applications.

Other extracts and preparation of gingkolides are known in the art, e.g., as described in Chen *et al.*, 1998, Bioorganic & Medicinal Chemistry Letters 8:1291-6.

The gingkolide compositions to be administered are in a form which maximizes ginkgolide bioavailability. For example, the composition is a variation of EGb 761 containing 27% ginkgo-flavonol glycosides, 7% terpene lactones. This composition extends bioavailability of pharmacologically active ginkgolide components (Li et al, 1997, *Planta Medica.* 63:563-5).

Among the compositions to be administered is BN 50730, an analog to the terpene trilactone BN52021 (GB). BN 50730 is a synthetic hetrazepine derivative of BN 52021. It shows a several ten-folds more potent PAF antagonistic activity *in vitro* than BN52021.

Anti-inflammatory drug combinations

The dose-response curve of astaxanthin in suppression of *in vitro* expression of an inflammation-associated cytokine was found to be favorably altered in the presence of a ginkgolide. Inflammatory damage is suppressed by astaxanthin or its derivatives and further reduced by co-administration of a ginkgolide.

The combination drug therapy regimen described herein is based on the pharmacological action of astaxanthin, ginkgolides and vitamin C. By acting as a powerful scavenger of free radicals, astaxanthin inhibits tissue damage mediated by these chemical species. However, since astaxanthin and its derivatives are primarily lipid-soluble, the adduct often remains membrane associated. Effective clearance of free radical-astaxanthin reaction products is mediated by co-administration of a water-soluble scavenger of free radicals. For example, the water-soluble free radical scavenger is vitamin C. Gingkolide compositions include extracts of ginkgo such as EGb761. The

gingkolide alone or in combination with vitamin C; or astaxanthin alone, or in combination with vitamin C; or astaxanthin plus a ginkgolide; or astaxanthin plus a ginkgolide plus vitamin C are used for suppression of disease-associated inflammation.

For example, the dose of astaxanthin plus ginkgolide and vitamin C required to achieve clinically significant suppression of inflammation is at least 5%, preferably at least 10%, preferably at least 25%, preferably at least 30%, more preferably at least 40%, and most preferably at least 50% less than that required for the same level of suppression of inflammation in the absence of a gingkolide and vitamin C. Suppression of inflammation is measured using methods known in the art, e.g., by detecting reduced expression of pro-inflammatory cytokines both *in vitro* (cell culture approach) and *in vivo* (immunohistochemical approach), given stimulus of experimental model in a manner known in the art to induce expression of these cytokines.

Toxicity

An astaxanthin/ginkgolide/vitamin C combination drug offers a method for achieving suppression of disease-associated inflammation in a manner superior to currently available drugs. Moreover, since each component exhibits low-to-negligible toxicity levels, and therefore, are applicable to a broad patient population.

Treatment and alleviation of symptoms of inflammatory disease

Clinical effects of formulations based on co-administration of astaxanthin plus ginkgolides and/or vitamin C include application to inflammation associated with autoimmune conditions (such as type I diabetes), asthma, psoriasis and cardiac disorders. These combinations will also aid in post-organ transplant drug therapy. Suppression of graft rejection-associated inflammation by these drugs is sufficient to maintain transplanted tissue in a healthy, functional state with little or no side effects.

Advantages of the invention include improved outcomes to transplant surgery (both in terms of survival as well as drug-related morbidity), decreased need for secondary hospitalization, and reduced expenditure of health care costs for transplant recipents.

The coadministration strategy also decreases the incidence of ischemia/reperfusion-related damage to organs occurring postoperatively, or as a result of

ischemic disease as a result of the capacity of these formulations to inhibit basic inflammatory processes.

Platelet Activating factor (PAF)/Calcium-dependent protection and mechanisms of inflammation

Cellular signaling pathways resulting in inflammatory responses are dependent largely upon receptor-mediated release of calcium stores (such as within the endoplasmic or sarcoplasmic reticulum), followed by expression of inflammatory mediators. This calcium availability may be reduced by treatment of a subject one or more subcomponents of *Ginkgo biloba* (e.g., EGb761). The gingkolide acts as an antagonist to the receptor for PAF, a potent bioactive phospholipid. The PAFR, when engaged by PAF, activates a signalling pathway causing a rise in intracellular calcium. Gingkolide compounds inhibit PAF-mediated increase in cytoplasmic calcium, in turn suppressing release of eicosonoids, pro-inflammatory cytokines, free radical species and other major mediators of inflammation.

Prevention of PAF/COX-2-mediated effects

PAF stimulates transcription of COX-2 (inducible prostaglandin synthase) which contributes to inflammatory damage. Ischemia of any tissue promotes PAF overproduction. PAF activity is blocked with ginkgolides exhibiting PAF receptor antagonist properties.

Amplification of pharmacological effect by increasing ginkgolide bioavailability

EGb 761 is a standardized extract of dried leaves of *Ginkgo biloba* containing 24% ginkgo-flavonol glycosides, 6% terpene lactones (24/6) such as ginkgolides A, B, C, J and bilobalide. The PAFR antagonistic and antioxidant effects of EGb761 confers clinical benefit, alone or when combined with astaxanthin and/or vitamin C. For example, an immunosuppressive compound contains a calcineurin inhibitor with extract of *Ginkgo biloba* with a ratio of 27% ginkgo-flavonol glycosides, 7% terpene lactones (27/7), enriched in ginkgolide B. Preparation of the gingkolide portion of the composition is known in the art, e.g., the method of Li, et al., 1997, Planta Medica. 63(6):563-5.

Therapeutic Administration

The results suggest that the combination of astaxanthin and a gingkolide is useful to inhibit inflammatory damage occurring as a result of a diverse range of diseases. The compositions are formulated into therapeutic compositions such as liquid solutions or suspensions, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable, eye drops or infusible solutions. The preferred form depends upon the mode of administration and the particular indication targeted. The compositions also include pharmaceutically acceptable vehicles or carriers. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. Actual methods of preparing such compositions are known to those skilled in the art (e.g., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., 18th edition, 1990).

The compositions are administered using conventional modes of delivery including intravenous, intraperitoneal, oral or subcutaneous administration. In addition to systemic administration, the compositions are locally administered, e.g., to the site of inflammation.

The dosages of astaxanthin and of gingkolide and vitamin C may vary depending on the severity and course of the disease, the patient's health and response to treatment, and the judgment of the treating physician.

Astaxanthin, vitamin C and the gingkolide are administered simultaneously or sequentially. Astaxanthin dosages range from 0.1 - 4.0 g/kg body weight per day; gingkolide compositions are administered in doses of 0.1 mg/kg/day to 1000 mg/kg/day. (e.g., 10 mg/kg/day - 60 mg/kg/day); and dosage of vitamin C will include regimens of 1.0 - 400.0 mg/kg/day. Routes of administration are comparable to those used for immunophilin-binding compounds such as calcineurin inhibitors.

The compositions are administered as prophylaxis to prevent onset of an inflammatory condition, or before or after development of disease. Subjects to be treated include those who have been diagnosed as having a condition characterized by aberrant immune activation (e.g., pathological T cell activation or pathological inflammation), those who are at risk of developing such a condition, and those who have a personal or family history of such a condition. Such aberrant inflammatory events include an asthma

attack. Methods for diagnosis are known in the art. For example, the anti-inflammatory compositions are useful to treat or prevent autoimmune disease and/or inflammatory conditions such as asthma, arthritis (e.g., rheumatoid arthritis, arthritis chronica progrediente and arthritis deformans) and rheumatic diseases. Specific auto-immune diseases for which the compositions of the invention may be employed include, autoimmune hematological disorders (including e.g. hemolytic anaemia, aplastic anaemia, pure red cell anaemia and idiopathic thrombocytopenia), systemic lupus erythematosus, polychondritis, sclerodoma, Wegener granulamatosis, dermatomyositis, chronic active hepatitis, myasthenia gravis, psoriasis, Steven-Johnson syndrome, idiopathic sprue, autoimmune inflammatory bowel disease (including e.g. ulcerative colitis and Crohn's disease) endocrine ophthalmopathy, Graves disease, sarcoidosis, multiple sclerosis, primary billiary cirrhosis, juvenile diabetes (diabetes mellitus type I), uveitis (anterior and posterior), keratoconjunctivitis sicca and vernal keratoconjunctivitis, interstitial lung fibrosis, psoriatic arthritis, glomerulonephritis (with and without nephrotic syndrome, e.g. including idiopathic nephrotic syndrome or minimal change nephropathy) and juvenile dermatomyositis.

Individuals to be treated include any member of the class Mammalia, including, humans and non-human primates, such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; and laboratory animals including rodents such as mice, rats and guinea pigs. Preferably, the mammal is not a rodent such as a rat. The compositions and methods are suitatble for treatment of adult, newborn and fetal mammals. Treatment encompasses the prevention of and adverse clinical conditions and the reduction or elimination of symptoms of a disease or adverse clinical condition. An anti-inflammatory composition refers to any composition that suppresses or prevents an undesired inflammatory response, e.g., prevents pain, tissue damage and disfigurement.

The combination drug therapy described herein utilizes astaxanthin and/or its derivatives; and a gingkolide composition, which contains PAFR antagonist activity and antioxidant activity. Preferably, the gingkolide compositions contains at least two antioxidant components of *Gingko biloba*, e.g., GB, GC, GJ, or GM, rather than one

component such as GM alone. For example, the gingkolide composition is Egb761 contains several antioxidant components of *Gingko biloba* in addition to a component with PAFR antagonist activity. EGb761 contains a full range of antioxidants and PAFR antagonists produced by leaves of the plant.

Anti- Inflammatory Combinations Useful for Protecting Ocular tissue from Imflammatoory or Oxidative Damage

The anti-inflammatory composition contains a carotinoid (e.g., astaxanthin or zeaxanthin) and a polyphenol. Optionally, the composition contains a glutathione precursor, a vitamin anti-oxidant an alpha lipoic acid or a trace mineral (hydromins). Additionally, the composition contains an inhibitor of stress induced mediated tissue damage, an inhibitor of pro-inflammatory prostaglandin (e.g., gamma linoleic acid or omega-3 fatty acid) or an inhibitor of NF*k*B (e.g., selenium, N-acetyl-L-cysteine, quercetin or bioflavonoids) or one or more essential vitamins or minerals. An essential vitamin or mineral is a vitamin or mineral that the body can not synthesize itself. These combination are herein refered to as "Ocular protective compositions or OPC combination"

A polyphenol is curcuma longo root powder, green tea, grape seed extract, citrus bioflavonoid. Alternatively, the polyphenol is a cox-2 inhibitor compound. Exemplary cox-2 inhibitor compounds include quercetin, bilberry extract, hops PE, blueberry powder or tart cherry powder.

A glutanthione precursor is for example taurine or N-acetyl-L-cysteine. Taurine is an amino acid-like compound and is a component of bile acids. Taurine are used to help absorb fats and fat-soluble vitamins. N-acetyl-L-cysteine is a free-radical scavenger.

A vitamin anti-oxidant include Vitamin A (e.g., beta carotene), Vitamin B, Vitamin C (e.g., ascorbic acid) Vitamin D, or Vitamin E. Vitamin B is a group of eight vitamins, which include thiamine (B1), riboflavin (B2), niacin (B3), pyridoxine (B6), folic acid (B9), cyanocobalamin (B12), pantothenic acid and biotin. Vitamin E is a mixture of tocopherols and tocotrienols.. Tococpherols include alpha-tocopherol, beta-tocopherol, gamma-tocopherol and delta-tocopherol. Tocotrienols include alpha-tocotrienol beta-tocotrienol gamma-tocotrienol delta-tocotrienol.

For example an anti- inflammatory composition contains a vitamin A palmitate compound, an ascorbic acid compound, a mixed tocopherol compound, an alpha lipoic compound, a polyphenol compound, an anthocyanidin compound, a blueberry compound, a ginko biloba compound, a hops PE compound, a quercetin compound, a tocotrienol complex compound, a N-acetyl -L-cysteine compound a curcuma longa root compound, zeaxznthin compound, an astaxanthin compound, and a tart cherry compound. Optionally, the compostion contains one or more of the following compounds, a beta carotene compound-alpha tocopheryl succinate compound. a pyridxine HCl compound, a folic acid compound, a zinc citrate compound, a grape seed extract compound, a citrus bioflavonoid compound, a taurine compound, zeaxznthin compound, a mixed carotenoid compound, and a hydromins compound a grape seed extract compound, a citrus bioflavonoid compound, a taurine compound, zeaxznthin compound, a mixed carotenoid compound,or a hydromins compound

Exemplary anti-inflammatory compositions include the formulations of Table A and B shown below.

TABLE A

LABEL CLAIM		INGREDIENT NAME	DAILY DOSAGE	
62.5	IU	Vitamin A (as Vitamin A Palmitate)	250.00	IU
1250	IU	Vitamin A (as Beta Carotene)	5000.00	IU
75	MG	Vitamin C (as Ascorbic Acid)	300.00	MG
60	IU	Vitamin D (as Cholecalciferol)	200.00	IU
25	IU	Vitamin E (as d-alpha Tocopheryl Succinate)	100.00	IU
12.5	IU	Vitamin E (as Mixed Tocopherols)	50.00	IU
0.015	MG	Vitamin K	0.06	MG
0.3	MG	Thiamin (as Thiamine Mononitrate)	120	MG
0.326	MG	Riboflavin	1.30	MG
4	MG	Niacin (as Niacinamide)	16.00	MG
0.75	MG	Vitamin B6 (as Pyridoxine HCl)	3.00	MG
0.1	MG	Folate (as Folic Acid)	0.40	MG
0.006	MG	Vitamin B12 (as Cyanocobalamin)	0.0024	MG
1.25	MG	Pantothenic Acid (as Calcium Pantothenate)	5.00	MG
0.0075	MG	Biotin	0.03	MG
68.75	MG	Choline (as Choline Bitartrate)	275.00	MG
0.025	MG	Chromium (as Chromium nicotinate)	0.10	MG
0.5	MG	Copper (as Copper Citrate)	2.00	MG
0.0375	MG	Iodine (as Potassium Iodine)	0.15	MG

25	MG	Magnesium (as Magnesium Citrate)	100.00	MG
0.575	MG	Manganese (as Manganese Citrate)	2.30	MG
0.025	MG	Selenium (as Selenomathionine)	0.10	MG
3.75	MG	Zinc (as Zinc Citrate)	15.00	MG
7.5	MG	Alpha Lipoic Acid	30.00	MG
50	MG	Green Tea (40% Polyphenois)	200.00	MG
1	MG	Bilberry Ext (25% Anthocyanidins)	4.00	MG
25	MG	Blueberry Powder	100.00	MG
62.5	MG	Ginkgo biloba SE 24/6	250.00	MG
2.5	MG	Hops PE	10.00	MG
12.5	MG	Quercetin	50.00	MG
12.5	MG	Tocotrienol Complex	50.00	MG
5	MG	Grape Seed Extract	20.00	MG
100	MG	Citrus Bioflavonoids	400.00	MG

LABEL CLAIM		INGREDIENT NAME	DAILY DOSAGE	
75	MG	Taunine	300.00	MG
50	MG	N-Acetyl-L-Cysteine	200.00	MG
12.5	MG	Curcuma longa Root Powder	50.00	MG
0.25	MG	Zeaxanthin	1.00	MG
0.25	MG	Astaxanthin	1.00	MG
12.5	MG	Mixed Carotenoids	50.00	MG
12.5	MG	Trace Minerals (Hydromins)	50.00	MG
12.5	MG	Tart Cherry Powder	50.00	MG
		Excipients		
56	MG	Croscarmellose Sodium	224.00	MG
14	MG	Magnesium Stearate	56.00	MG
24	MG	Silicon Dioxide	96.00	MG
28	MG	Stearic Acid	112.00	MG
210	MG	Microcrystalline Cellulose	840.00	MG
40.16	MG	Color Coating (3% target weight)		

TABLE B

LABEL CLAIM		INGREDIENT NAME	DAILY DOSAGE	
125	IU	Vitamin A (as Vitamin A Palmitate)	250.00	IU
2500	IU	Vitamin A (as Beta Carotene)	5000.00	IU
150	MG	Vitamin C (as Ascorbic Acid)	300.00	MG
50	IU	Vitamin E (as d-alpha Tocopheryl Succinate)	100.00	IU
25	IU	Vitamin E (as Mixed Tocopherols)	50.00	IU
1.6	MG	B6 (as Pyndoxine HCl)	3.00	MG
0.2	MG	Folate (as Folic Acid)	0.40	MG
7.5	MG	Zinc (as Zinc Citrate)	15.00	MG
15	MG	Alpha Lipoic Acid	30.00	MG
100	MG	Green Tea (40% polyphenois)	200.00	MG

0.5	MG	Astaxanthin	1.00	MG
125	MG	Ginkgo biloba SE 24/6	250.00	MG
5	MG	Hope PE	10.00	MG
25	MG	Quarcetin	50.00	MG
25	MG	Tocotrienol Complex	50.00	MG
2	MG	Bilberry Ext (25% Anthocyanidins)	4.00	MG
25	MG	Blueberry Powder	50.00	MG
25	MG	Tart Cherry	50.00	MG
25	MG	Curcuma longa Root Powder	50.00	MG
100	MG	N-Acetyl-L-Cysteine	200.00	MG
		Excipients		
48	MG	Croscarmellose Sodium	96.00	MG
12	MG	Magnesium Staearaaate	24.00	MG
14	MG	Silicon Dioxide	28.00	MG
25	MG	Stearic Acid	50.00	MG
200	MG	Microcrystalline Cellulose	400.00	MG
35.34	MG	White Coating 3% target weight)		

Reduction of Ocular Inflammation and Treatment of Ocular Inflammatory Disease

Inflammation is inhibited by administering to tissue an OPC combination described above. Optionally, the tissue is contacted with a omega -3- fatty acid such as eicosapentaenoic acid or docosahexaenoic acid.

Tissues to be treated include ocular tissue such as sclera tissue, iris tissue, cornea tissue, pupil tissue, lens tissue, conjuctiva tissue, vitreous tissue, choroids tissue, macula tissue or retina tissue. Inhibition of inflammation is characterized by a reduction of redness, pain and swelling of the treated tissue compared to a tissue that has not been contacted with an OPC combination. OPC combinations are administered in an amount sufficient to decrease (e.g., inhibit) inflammatory cytokine production. An inflammatory cytokine is a cytokine that modulates, e.g., induces or reduces an inflammatory response. An inflammatory response is evaluated by morphologically by observing tissue damage, localized redness, and swelling of the affected area. An inflammatory cytokine is a proinflammatory cytokine. For example the inflammatory cytokine is, TNF alpha, interferon (e.g., alpha, beta or gamma), or interleukin (e.g., IL-1, IL-6, IL-10, IL-12, IL-14, IL-18). Cytokines are detected for example in the serum, plasma or the tissue. Cytokine production is measured by methods know in the art.

Tissues are directly contacted with an OPC combination. For example the OPC combination is applied directly to the eye. Alternatively, the OPC combination is administered systemically. An inflammatory response is evaluated by morphologically by observing tissue damage, localized redness, and swelling of the affected area.

The methods are useful to alleviate the symptoms of a variety of ocular inflammatory disorders. The ocular inflammatory disorder is acute or chronic. Ocular inflammatory disorders include dry eye, or macular degeneration.

The methods described herein lead to a reduction in the severity or the alleviation of one or more symptoms of an ocular inflammatory disorder such as those described herein. Ocular inflammatory disorders are diagnosed and or monitored, typically by a physician using standard methodologies. Alleviation of one or more symptoms of the ocular inflammatory disorder indicates that the compound confers a clinical benefit.

Dry eye syndrome is one of the most common problems treated by eye physicians. Over ten million Americans suffer from dry eyes. It is usually caused by a problem with the quality of the tear film that lubricates the eyes. Dry eye syndrome has many causes. One of the most common reasons for dryness is simply the normal aging process. As we grow older, our bodies produce less oil – 60% less at age 65 then at age 18. This is more pronounced in women, who tend to have drier skin then men. The oil deficiency also affects the tear film. Without as much oil to seal the watery layer, the tear film evaporates much faster, leaving dry areas on the cornea. Other factors, such as hot, dry or windy climates, high altitudes, air-conditioning and cigarette smoke also cause dry eyes. Contact lens wearers also suffer from dryness because the contacts absorb the tear film, causing proteins to form on the surface of the lens. Certain medications, thyroid conditions, vitamin A deficiency, and diseases such as Parkinson's and Sjogren's also cause dryness. Women frequently experience problems with dry eyes as they enter menopause because of hormonal changes.

Symptoms of dry eye include itching, burning irritation, redness, blurred vision that improves with blinking, excessive tearing, increased discomfort after periods of reading, watching TV, or working on a computer.

There are several methods to test for dry eyes. For example, the underlying cause of the dry eyes will be determined by measuring the production evaporation rate and quality of the tear film. Special drops that highlight problems that would be otherwise invisible are particularly helpful to diagnose the presence and extent of the dryness.

Macular degeneration is a degenerative condition of the macula (central retina) It is the most common cause of vision loss in the United States in those 50 or older, and its prevalence increases with age. Macular degeneration is caused by hardening of the arteries that nourish the retina. This deprives the sensitive retinal tissue of oxygen and nutrients that it needs to function and thrive. As a result, the central vision deteriorates.

Macular degeneration varies widely in severity. In the worst cases, it causes a complete loss of central vision, making reading or driving impossible. For others, it may only cause slight distortion. Macular degeneration does not cause total blindness since it does not affect the peripheral vision.

Macular degeneration is classified as either wet (neovasular) or dry (non-neovasular) About 10% of patients who suffer from macular degeneration have wet AMD. This type occurs when new vessels form to improve the blood supply to oxygen-deprived retinal tissue. However, the new vessels are very delicate and break easily, causing bleeding and damage to surrounding tissue. Macular degeneration is caused by variety of factors. Genetics, age, nutrition, smoking, and sunlight exposure all play a role.

Symptoms of macular degeneration include loss of central vision, difficulty reading or performing tasks that require the ability to see detail, distorted vision Eye physicians usually diagnose macular degeneration. Methods of diagnosis, include for example, vision testing amsler grid test, ophthalmoscopy, fundus photography, and fluorescein angiography.

The OPC combinations are formulated into therapeutic compositions. The compositions are administered using conventional modes of delivery including intravenous, intraperitoneal, oral or subcutaneous administration. Additionally the compositions are locally administered, e.g., to the eye.

The dosages of OPC combinations vary depending on the severity and course of the ocular inflammatory disorder. Typically, a dosage regimens includes one or two tablets administered orally twice a day. Preferably the OPC therapeutic compositions are administered with omega-3 fatty acids. Alternatively, the omega-3-fatty acids are administer prior to or after administration of the OPC therapeutic compositions.

Reactive oxygen free radicals and the pathogenesis of dry eye

Reactive oxygen species (ROS) expressed primarily by leukocytes infiltrating affected tissue are major mediators of tissue damage in inflammation. Hence antioxidants suppress these effects. A major etiologic factor in the age-related form of dry eye is endogenous lipophilic and cationic compound N-retinyl-N-retinylidene ethanolamine (A2E) which mediates formation of reactive oxygen and nitrogen free radical species (Shaban, H., 2002). ROS are substantially upregulated by T lymphocytes during activation; moreover blocking this enhancement with antioxidants such as glutathione may suppress the activation process, (Vint, IA, 1993, Vint IA, 1994). *Ginkgo biloba* contains several compounds with known antioxidant capability including Ginkgolide B, C, J and M and Bilobalide. Astaxanthin is one of the strongest naturally occurring free radical scavengers known (Wang X, 2000, Fan L, 1998, Kurashige M, 1990, Morrow J, 1995), with the additional benefit of low toxicity combined with a capacity to stabilize normal immune activity (Jyonouchi H, 1994, Jyonouchi H, 1996, Okai Y, 1996).

Phospholipase A2 (PLA-2) and the pathogenesis of dry eye

The action of PLA-2 on membrane lipids of macrophages and PMNs during inflammatory processes causes activation of COX-2 and inducible nitric oxide synthetase (iNOS), both of which contribute to tissue destruction and pain in dry eye The biflavone gingkgolides bilobetin and ginkgetin are potent inhibitors of PLA-2 and thus are expected to contribute to the observed capacity of *Ginkgo biloba* to ameliorate dry eye (Baek, S., *et al* 1999).

Cytokine- inducible nitric oxide synthetase (iNOS) and the pathogenesis of dry eye

Among the major cytokine-mediated effects is expression of inducible nitric oxide synthase (iNOS/NOS-2) by activated macrophages at high levels and at lower but still

significant levels by several secretory (including lacrimal gland) epithelial cell types under the influence of TNF-α and IL-1β, causing increased nitric oxide, a process may be a significant pathophysiological pathway of dry eye syndrome (Beauregard C, 2003). The biflavone gingkgolides bilobetin and ginkgetin are potent inhibitors of iNOS via their inhibitory effect on phospholipase A-2 (PLA-2) and thus are expected to contribute to the observed capacity of *Ginkgo biloba* to ameliorate dry eye (Baek, S., *et al* 1999).

Inducible cyclooxygenase (COX-2) and the pathogenesis of dry eye

Ocular inflammation in dry eye occurs in part due to breach of the blood-ocular barrier and the attraction of macrophages, PMNs and other leukocytes to affected tissue. This process is mediated substantially by release of inflammatory metabolites such as prostaglandins both from ocular tissue and from emigrant leukocytes. A major contributor to this process is the inflammation-induced enzyme cyclooxygenase-2 (COX-2) which has been demonstrated in many ocular tissues including corneal epithelium and endothelium and pigmentary epithelium) (Guex-Crosier, Y., 2001). Moreover inhibitors of COX-2 in clinical practice have been demonstrated to ameliorate ocular inflammation and pain as will as contributing to maintenance of a good mydriasis during surgery and control of postoperative cystoid macular edema (Guex-Crosier Y., 2001). The biflavone gingkgolides bilobetin and ginkgetin are potent inhibitors of COX-2 via their inhibitory effect on phospholipase A-2 (PLA-2) and thus contribute to the observed capacity of *Ginkgo biloba* to ameliorate dry eye (Baek, S., *et al* 1999).

Eosinophils and cAMP-phosphodiesterase and the pathogenesis of dry eye

Eosinophils are a major component of the inflammatory infiltrate characteristic of dry eye and a major contributor to inflammatory damage in the disorder (Lobefalo L. 1999). It has been shown in a histamine-induced guinea pig eye model of tissue eosinophilia, oral treatment of the animals with rolipram, an isozyme IV-selective inhibitor of cAMP-specific phosphodiesterase significantly suppressed infiltrate of these cells (Newsholme SJ, 1993). The biflavone ginkgolides also exhibit varying capacity to inhibit cAMP-phosphodiesterase, with the degree of enzyme inhibition following the order: amentoflavone > bilobetin > sequoiaflavone > ginkgetin = isoginkgetin; but

almost no capacity for inhibition of this enzyme by sciadopitysin (Saponara R., et al, 1998).

Example 1: Administraton of astaxanthin leads to suppression of inflammation

In vitro studies indicate that astaxanthin suppresses expression of inflammation-associated T cell surface antigens in PMA/I-treated human PBMC. Cells isolated from whole blood of healthy volunteers were cultured in 96-well plates (2 X 10^6/ml) for 24 hours in RPMI 1640, with phorbol 12-myristate 13-acetate (PMA: 25 ng/ml) in conjunction with ionomycin, or media; or with PMA/I plus astaxanthin (10^{-5} M).

Following incubation, cultured cells were immunofluorescently labeled with monoclonal antibodies specific for CD3 (T lymphocytes) and the cell surface antigens CD25 and CD54 which are known to be upregulated *in vivo* during both immune activation and during inflammatory processes. Analysis of blood for representation by selected lymphocyte subpopulations was conducted by two-color flow cytometry. Astaxanthin alone significantly inhibited each of the activated T cell phenotypes (Table 1A and 1B).

Table 1A (Subject A)

Stimulation Conditions	%CD3+CD54+ cells	%CD3+CD54+ cells
Unstimulated	1.7	2.7
PMA/I	56.3	66.3
Astx 10-7 M	8.4	10.6
Astx 10-6 M	4.2	3.3

Table 1B (Subject B)

Stimulation Conditions	%CD3+CD54+ cells	%CD3+CD54+ cells
Unstimulated	2.2	1.6
PMA/I	49.5	54.6
Astx 10-7 M	16.2	21.9
Astx 10-6 M	6.3	10.3

Example 2: Expression of TNF-α by human PBMC *in vitro* is suppressed by astaxanthin but not BN52021 and is suppressed maximally with astaxanthin plus BN52021.

PBMC (2 X 10^6/ml) from 2 donors were stimulated with 50 μg/ml, PHA; or with astaxanthin (10^{-6} M); or with the ginkgolide BN52021 (GB) (10^{-4} M); or with a combination of astaxanthin (10^{-6} M) plus GB (10^{-4} M); or with media. Cells were cultured 24 hours at 37° C, 5% CO_2 and analyzed by ELISA for supernatant concentration of TNF-α. Each data point is the mean of triplicate samples. Results show that TNF-α expression by PBMC was significantly increased relative to unstimulated control cultures as a result of PHA stimulation; and was suppressed by astaxanthin, but not GB treatment. As shown in Table 2, the combined treatment with both astaxanthin and GB suppressed expression of this pro-inflammatory cytokine below that of astaxanthin alone.

Table 2

Stimulation Conditions	TNF- alpha pg/ml	Standard Deviation
Unstimulated	164.52	21.73
PHA, 50 μg/ml	1676.75	103.57
Astx 10^{-6} M	781.34	186.17
BN52021 10^{-4} M	1689.21	218.15
Astx 10^{-6} M + BN52021 10^{-4} M	225.76	52.97

Example 3: Compositions containing a biflavonoid ginkgolide, astaxanthin, vitamin C, and/or an NSAID suppress onset of Alzheimer's disease

Elevation of intracellular cAMP increases the recovery of APP alpha, the physiological alpha-secretase-derived product of beta APP processing, and concomittantly lowers the production of the pathogenic beta/gamma-secretase-derived A beta fragment (A42). The pathogenesis of Alzheimer's disease correlates with altered production, aggregation and deposition in neuronal tissue of of the A peptide, a proteolytic fragment of 40–42 residues derived from APP. The longer isoform, A42, is selectively increased in the disease and its presence promote production of beta-amyloid deposits. Beta amyloid in turn induces free radical production, increased glucose uptake, apoptosis and death of nerve cells. Extract of Ginkgo biloba (EGb 761) inhibits, in a dose-dependent manner, the formation of beta-amyloid-derived diffusible neurotoxic

soluble ligands (ADDLs) involved in the pathogenesis of Alzheimer's disease. The mechanism for this protective effect involves elevation of neuronal cAMP which occurs as a result of the cAMP phosphodiesterase-inhibitory properties of the biflavonoid components of Ginkgo biloba.

NSAIDs ibuprofen, indomethacin and sulindac sulphide preferentially decrease the highly amyloidogenic A42 peptide (the 42-residue isoform of the amyloid- peptide) produced from a variety of cultured cells by as much as 80% independently of COX activity. Significant gastrointestinal and renal toxicity associated with long-term COX-1 inhibition limit the clinical utility of current NSAIDS as A42-lowering agents. Because the A42 effect is independent of COX activity, compounds (e.g., the combinations described herein) with optimized A42 reduction and little to no inhibition of COX-1 activity are useful for the prevention or alleviation of symptoms associated with Alzheimer's Disease. Such agents represent a new generation of 'anti-amyloid' drugs that selectively target production of the highly amyloidogenic A42 species without inhibiting either COX activity or the vital physiological functions.

Sustained high dosage, non-steroidal, anti-inflammatory drugs (NSAIDs) inhibit onset of Alzheimers disease, but the dosage required to suppress the disease is toxic.

Biflavonoid components of ginkgo biloba represent a new generation of anti-amyloid drugs which, when used in combination with NSAIDs lower the effective NSAID dosage to subtoxic levels, thereby enabling them to be used to prevent Alzheimer's disease at little or no risk to the general health of the patient.

Astaxanthin and vitamin C contribute to suppression of alzheimers disease in a manner synergistic with combiniations of ginkgo biflavoinoids by suppressing disease associated inflammation, primarily as free radical scavengers.

The compositions described herein are useful to prevent onset of Alzheimers disease by inhibiting formation of Beta amyloid plaques as a result of the combined action of the NSAIDs ibuprofen, indomethacin and sulindac sulphide, (and/or other drugs which act through COX-2 inhibition); plus the biflavonoid ginkgolides: amentoflavone, bilobetin, sequoiaflavone, ginkgetin and isoginkgetin. Inflammation associated with Alzheimers is suppressed by combining NSAID + ginkgolide formulations with

astaxanthin and vitamin C. The combined action of the lipid-soluble carotenoid (principally astaxanthin) with vitamin C and one or more components of a Ginkgo biloba extract, in combination with NASAIDs mediates prevention or suppression of disease-associated inflammation. The combination drug therapy described herein utilizes astaxanthin and/or its derivatives; and a gingkolide composition, which contains cAMP phosphodiesterase-inhibitory capabilities.

Example 4: Compositions of ginkgolides, astaxanthin, plus vitamin C, potentiate anti-asthmatic effects of cetirizine

Cetirizine compounds, e.g., Zyrtec™ (cetirizine hydrochloride), inhibit histamine release by mast cells. Histamine release occurs when mast cells are stimulated, e.g., when antibodies interact with their surface H1 receptors (H1R). Selective inhibition of H1R by Zrytec prevents downstream events which include intracellular calcium ion release and calcium uptake and protein kinase C translocation. H1 inhibition inhibits these effects and also promotes the activation of adenylate cyclase and the resulting accumulation of cAMP.

Components of Ginkgo biloba include terpene antagonists of PAF receptors (PAFR) which synergize with cetirizine and other histamine release blockers in reducing the calcium signal (a consequence of PAFR stimulation). Biflavonoid ginkgolides further reduce the effective dosage of cetirizines by their inhibition of cAMP phosphodiesterase, an effect which allows augmented accumulation of cAMP.

Astaxanthin also potentiates the effect of cetirizines. Histamine release from mast cells is significantly reduced by antioxidants, and astaxanthin further contributes to potentiation of the pharmacological activity of cetirizines.

The compositions described herein are useful to augment the therapeutic activity of cetirizines such as Zyrtec™, while reducing its effective dosage. For example, such composition contain a cetirizine compound plus terpene trilactones, such as Gingkolide A, Gingkolide B, Gingkolide C, Gingkolide J, Gingkolide M and bilobalide; or the biflavonoid ginkgolides: amentoflavone, bilobetin, sequoiaflavone, ginkgetin and isoginkgetin. The combined action of the lipid-soluble carotenoid (e.g., astaxanthin) with vitamin C and one or more components of a Ginkgo biloba extract, mediates prevention

or suppression of disease-associated inflammation. The combination drug therapy described herein utilizes astaxanthin and/or its derivatives; and a gingkolide composition, which contains cAMP phosphodiesterase-inhibitory as well as antioxidant capabilities.

Cetirizine compounds such as Zyrtec™ are antihistamines useful in general treatment of allergies, especially seasonal or perennial rhinitis and chronic urticaria. The risk of toxicity associated with such compounds is substantially increased in individuals with kidney impairment, in particular geriatric patients. The combination drug therapy regimen (e.g., cetirizine administered with astaxanthin and/or a gingkolide), reduces the effective dosage necessary for a beneficial clinical outcome.

The lipid antioxidant astaxanthin and the terpene and biflavonoid components of *ginkgo biloba* synergize with a cetirizine such as Zyrtec™ to reduce H1-mediated histamine release by mast cells and other tissue. The synergistic effect of this combination permits a reduction in the effective dosage of a cetirizine needed to achieve a desired therapeutic outcome, thereby reducing adverse side effects of a cetirizine compound.

When cells were cultured in the presence of Ginkgolide B (GB) plus cetirizine, the PMA/Ionomycin-induced expression of the T cell activation antigen CD25 was suppressed to levels below that mediated by either GB or Zrytec alone.

Astaxanthin or astaxanthin plus a cetirizine together was administered to an allergy patient. Astaxanthin alone did not result in alleviation of allergy symptoms. However, the therapeutic effect of the combination (an antioxidant such as astaxanthin and cetirizine) exceeded that of either agent alone (as measured by reduction of allergry symptoms such as itching).

Example 5: Suppression Of Lymphocyte Activation By Citirazene And Azalestine

Experiements were carried out to determine whether the immunoregulatory capacity of two commonly-used H1-inhibitory antihistamines: cetirizine dihydrochloride (CTZ/Zyrtec) and azelastine (AZE/Astelin) is potentiated by the platelet activating factor receptor (PAFR) antagonist and free radical scavenger Ginkgolide B (GB). For these studies, peripheral blood mononuclear cells (PBMC) from asthma patients, which were cultured 24 hours with either 50 μg/ml PHA or PHA plus selected dosages of each drug

were analyzed by 3-color flow cytometry for expression of CD25+ and HLA-DR+ on CD3+ (T cells). The results shown in Table 3 are reported as stimulation indices (SI) of %CD3+CD25+ cells in cultures treated with PHA alone to %CD3+CD25+ cells in each drug-supplemented culture. Each drug was first evaluated independently over a 3-log dose range from 10^{-8}-10^{-6} M. Maximal suppression of activation was observed at 10^{-8} M, where CTZ caused a 29% decrease in SI for CD25+ (p=0.024); and 53% for HLA-DR (p=0.009); with AZE resulting in decreases of 19% for CD25+ (p=0.33); and 45% for HLA-DR (p=0.001); and GB 10^{-8} M suppressing HLA-DR+ by 39% (p=0.01). When compared to effects at 10^{-8} M, each drug at 10^{-7} M showed reduced capacity to independently suppress PHA-mediated induction of the two activation antigens. However at this concentration, GB was observed to augment the capacity of CTZ to suppress expression of CD25+ (p=0.003) and HLA-DR (p=0.004). The suppressive effect of AZE at 10^{-7} was also potentiated by GB at the same concentration in the case of CD25+ (p=0.014) and HLA-DR (p=0.000). The data indicated that GB improved the pharmacological activity of CTZ and AZE at a concentration of 10^{-7} M for each of the three components. These data indicate that GB-augmented antihistamine formulations are useful to alleviate a symptom of asthma-associated inflammation, e.g., abnormal T cell activation.

Table 3: Effect of cetirizine/Zyrtec (CTZ), or azalestene (AZE) on induction of CD25+ (CD3+CD25+) and HLA-DR+ (CD3+HLA-DR+) T lymphocytes in human peripheral blood mononuclear cells (PBMC)

Culture	SI CD25	P vs Stim	N subjects	SI HLA-DR	P vs Stim	N subjects
Unstimulated	0.09 ± 0.03	0.000	20	0.450 ± 0.09	0.000	6
Stimulated	1.00 ± 0.00	--	20	1.00 ± 0.00	--	5
CTZ 10^{-8} M	0.71 ± 0.12	0.024	8	0.47 ± 0.13	0.009	5
CTZ 10^{-7} M	0.88 ± 0.14	0.060	20	0.58 ± 0.11	0.010	5
CTZ 10^{-6} M	0.93 ± 0.06	0.141	20	0.70 ± 0.04	0.001	5
AZE 10^{-8} M	0.81 ± 0.08	0.033	8	0.55 ± 0.09	0.001	5
AZE 10^{-7} M	0.88 ± 0.08	0.092	19	0.56 ± 0.10	0.006	5
AZE 10^{-6} M	1.13 ± 0.15	0.210	17	0.61 ± 0.13	0.018	5
GB 10^{-8} M	0.81 ± 0.14	0.105	8	0.61 ± 0.10	0.010	5
GB 10^{-7} M	1.18 ± 0.21	0.196	14	0.62 ± 0.18	0.051	5

GB 10^{-6} M	0.94 ± 0.12	0.308	15	0.72 ± 0.18	0.100	5
GB 10^{-8} M + CTZ 10^{-8} M	0.79 ± 0.08	0.038	8	0.62 ± 0.13	0.020	5
GB 10^{-8} M + CTZ 10^{-7} M	0.88 ± 0.08	0.096	8	0.75 ± 0.13	0.630	5
GB 10^{-8} M + AZE 10^{-8} M	0.86 ± 0.09	0.091	8	0.61 ± 0.04	0.001	5
GB 10^{-8} M + AZE 10^{-7} M	0.71 ± 0.12	0.024	7	0.53 ± 0.17	0.035	4
GB 10^{-7} M + CTZ 10^{-8} M	0.73 ± 0.07	0.002	8	0.64 ± 0.10	0.012	5
GB 10^{-7} M + CTZ 10^{-7} M	0.65 ± 0.09	0.003	8	0.51 ± 0.11	0.004	5
GB 10^{-7} M + AZE 10^{-8} M	0.73 ± 0.12	0.033	8	0.62 ± 0.11	0.014	5
GB 10^{-7} M + AZE 10^{-7} M	0.71 ± 0.10	0.014	8	0.50 ± 0.04	0.000	5

Example 6: Effects Of Astaxanthin and Ginkgolide B On T Lymphocyte Activation

Experiments were carried out to determine whether formulations based on the platelet activating factor receptor (PAFR) antagonist and free radical scavenger Ginkgolide B (GB) in combination with the antioxidant carotenoid astaxanthin (ASX) suppress T cell activation in the same dose range as two commonly-used antihistamines: cetirizine dihydrochloride (CTZ/Zyrtec) and azelastine (AZE/Astelin). Peripheral blood mononuclear cells (PBMC) from asthma patients were cultured 24 hours with either 50 μg/ml PHA or PHA plus selected dosages of each drug and analyzed by 3-color flow cytometry for expression of CD25+ on CD3+ (T cells). Results are reported as stimulation indices (SI) of %CD3+CD25+ cells in cultures treated with PHA alone to %CD3+CD25+ cells in each drug-supplemented culture. Formulations which significantly reduced SI of PHA-treated cells ranked in order of increasing magnitude of suppression are as follows: ASX 10^{-7} M < GB 10^{-8}M + ASX 10^{-8}M < GB 10^{-8}M < GB10^{-7}M + ASX 10^{-7}M < GB 10^{-8}M + ASX 10^{-7}M ASX < CTZ 10^{-5}M < GB 10^{-6}M< GB 10^{-7}M + ASX 10^{-8}M < AZE 10^{-5}M. The data indicate that suppression of T cell activation below fully-stimulated values by GB, ASX and their combinations was comparable and for some combinations better than that mediated by CTZ and AZE.

The studies were carried out as follows.

Patients

Subjects for this study included 12 patients diagnosed with atopic asthma, 7 male and 5 female, ranging in age from 21 to 40 years (mean 28 ± 1.8 years). Disease duration ranged from 2 to 12 years. Atopy was defined on the basis of one or more positive skin prick tests to a range of 20 allergens. None of the patients had received systemic therapy

for at least 6 weeks prior to blood collection. The mean serum IgE was 335 (170-480) IU/ml.

Cell Cultures

Venous blood for each subject was collected in polyethylene tubes containing EDTA during a one hour morning time interval. PBMC were separated by Ficoll-paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Cells were washed and suspended in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) at density of 1 X 10^6 cells/ml. PBMC were stimulated with 50 μg/ml Phytohemaglutinin (PHA) (Sigma Immunopharmaceuticals, St. Lous Mo.), or PHA plus 10^{-8}-10^{-5} M astaxanthin (Natural Alternatives International (NAI) Inc., San Marcos CA); or ginkgolide B 10^{-8}-10^{-6} M (NAI San Marcos CA); or selected combinations of ASX plus GB. Comparison of ASX and GB effects on T cell activation were made with two other pharmacological agents with anti-asthmatic properties by treating cells with 10^{-7}-10^{-4} M cetirizine dihydrochloride (Pfizer Pharmaceuticals, Norwich CT); or 10^{-7}-10^{-4} M azalestine hydrochloride (Wallace Pharmaceuticals, Somerset NJ), followed by evaluation of cultures for the same biological endpoints as ASX/GB-treated cells. Each reagent was added at the outset of a 24 hours culture period, followed by harvest of cellular fraction for immunophenotyping studies.

Flow cytometric analysis

Cells harvested from cultures by centrifugation were incubated for 30 min at 4^{o} C with 10 μl each of flourescein-isothiocyanate (FITC)-CD3 and phycoerythrin (RD1)-CD25 conjugated monoclonal antibodies (mAb) (Dakopatts, A/S, Glostrup, Denmark), followed by fixation with paraformaldehyde. Two-color Flow cytometry was conducted using a Coulter Epics XL automated flow cytometer (Coulter Scientific, Hialeah, FL, USA). Isotypic controls for the monoclonal antibodies (mAb) used to detect antigens of interest were established for each cell preparation. Positive analysis regions for cells expressing specific surface markers were set against controls and specific binding of fluorophore-conjugated mAb was analyzed by cytofluorography according to standard methods recommended by the manufacturer. Lymphocyte subpopulations were identified

by position on forward and side scatter plots and live-gated. Expression of each antigen was reported as percentage cells positive for a particular T cell subpopulation defined by expression of CD3 (T lymphocyte marker) plus CD25, plus or minus standard error.

Statistical analysis

Statistical analysis was performed using an independent *t*-test. All statistical analyses were performed using the SPSS for Windows statistical package (Norusis/SPSS, Inc.). A value of $p < 0.05$ was considered statistically significant

T lymphocyte activation

Culture of PBMC for 24 hours with 50 μg/ml of the immunostimulatory lectin PHA resulted in significant activation of T lymphocytes, measured as increased percentage of CD3+CD25+ cells versus unstimulated cultures (Table 3). The capacity of formulations evaluated in this study to suppress immune activation was measured as a stimulation index (SI), defined as the ratio of CD3+CD25+ cells in each test condition to CD3+CD25+ in cultures treated with PHA alone. Assigning fully-stimulated cultures an SI value of 1.00, we observed that 9 of the 26 candidate formulations resulted in significant ($p<0.05$) reduction in SI (Table 1).

Effects of astaxanthin and GB on T lymphocyte activation

As shown in Fig. 1, stimulation indices for PHA-treated cells were suppressed significantly by astaxanthin at a concentration of 10^{-7} M (SI = 0.89±0.06, $p<0.034$). Ginkgolide B significantly reduced SI of PHA-stimulated cells at dosages of 10^{-6}M (SI = 0.77±0.12, p=0.048); and 10^{-8}M (SI = 0.86±0.07, p=0.05) (Fig. 2). Combinations of these agents also significantly suppressed immune activation. These formulations included 10^{-7}M GB in combination with 10^{-7}M ASX (SI = 0.86±0.06, p=0.037); 10^{-7}M GB + 10^{-8}M ASX (SI = 0.77±0.05, p=0.006); 10^{-8} M GB + 10^{-7} M ASX (SI = 0.85±0.05, p=0.015); and 10^{-8}M GB + 10^{-8}M ASX (SI = 0.87±0.06, p=0.040) (figure 3); and cells stimulated with a combination of 10-8M ASX plus 10^{-7}M ginkgolide B, which suppressed induction of CD3+CD25+ cells to an SI of 0.77±0.05, significantly below the suppression mediated by 10^{-8}M ASX alone acting on PHA-stimulated cultures (p=0.051)

(Figs. 1 and 3). Nevertheless treatment of cells with 10^{-7}M GB + 10^{-8}M ASX failed to significantly suppress activation below 10^{-7}M GB alone acting on PHA-treated cells (p=0.373) (Figs. 2 and 3).

Effects of cetirizine and azelastine on T lymphocyte activation

Two commonly-used anti asthmatic compounds, cetirizine dihydrochloride (Zyrtec, CTZ) and azelastine HCl (Astelin, AZE) were evaluated under the same conditions as ASX and GB for their ability to suppress T cell activation. Cells treated with PHA exhibited significant reduction in induction of CD3+CD25+ cells at a concentration of 10^{-5}M for both CTZ(SI = 0.78±0.11, p=0.05) (Fig. 4A); and AZE (SI = 0.76±0.12, p=0.034) (Fig. 4B).

Combination drug therapy for inhibition of T cell activation in asthma subjects

Asthma is associated with elevated expression in bronchoalveolar tissue of Th2 cytokines (IL-3, IL-5, and GM-CSF), which in turn upregulate eosinophil recruitment, activation, proliferation and differentiation, promoting tissue injury and fibrosis via an increased production of a variety of toxic metabolites. Histamine release blockers such as azalestine and cetirizine which treat the disease downstream from the underlying pathogenic T lymphocyte activity have been successful in partially alleviating its symptoms, but are often not as effective as agents which directly suppress abnormal T cell activation. Nevertheless since cellular signalling pathways which promote tissue damage in asthma, exert positive feedback and increase T cell activation, drugs which inhibit release or activity of inflammatory metabolites are also expected to exhibit immunosuppressive properties. Indeed, the H1 receptor antagonist terfenadine is observed to inhibit proliferation and expression of IL-4 and IL-5 production by anti-CD3/-CD28 and PMA-activated human T cells *in vitro*. Since both of these Th2 cytokines are implicated as major factors in asthma pathogenesis, therapeutic effects of this drug are likely mediated at least in part by suppression of T cell activity.

Ginkgolide B and astaxanthin with azalestine and cetirizine were tested for the ability to suppress T cell activation in PHA-stimulated cultures of human PBMC taken from asthma patients. These experiments were designed with the recognition that suppression of T lymphocyte activation is not the primary mechanism by which each

compound mediates its therapeutic effects. However, since T cell activity is a critical component of the cascade of signaling events resulting in the symptoms of asthma, T cell suppression represents a useful index to gauge the relative effectiveness of the pharmacological agents tested. Table 3 shows the effect of each stimulation condition on cells with respect to their ability to inhibit PHA-induced upregulation of the IL-2 receptor (CD25) on CD3+ cells (an index of T cell activation). When astaxanthin alone was added to PHA-treated cultures, significant suppression of T lymphocyte activation occurred at a concentration of 10^{-7}M (Fig. 1); whereas SI values significantly lower than 1.00 (fully stimulated) were observed over a 3 log dose range of ginkgolide B, with SI values significantly less than 1.00 observed at GB concentrations of 10^{-8} M and 10^{-6} M (Fig. 2). When combinations of ASX and GB were evaluated for their capacity to suppress T cell activation, four combinations of the compounds were observed to result in significant reduction in PHA-mediated induction of CD3+CD25+ cells; with an optimal combination occurring at a concentration of 10^{-8} M ASX plus 10^{-7} M GB (Fig. 3). Mechanisms contributing to suppression of T cell activation by ASX, GB and their combination are likely a consequence of the major biochemical properties of each compound acting together. Reactive oxygen species (ROS) are substantially upregulated by T lymphocytes during PHA-mediated activation; moreover blocking this enhancement with antioxidants alters the activation process.

Although previous studies of cetirizine suggest that it has no significant effect on T cells, the data described herein indicate that at an optimal concentration of 10^{-5} M, it will suppress at least those aspects of T cell activation involving expression of CD25 (Fig. 4A). Cetirizine also displays an ability to downregulate aspects of T cell activation related to chemotaxis. The present results indicate that astaxanthin and ginkgolide B act in concert to mediate antiasthmatic effects as well or better than currently-used medications. Compositions containing the combination of compounds described herein reduce inflammation (e.g., by inhibiting T cell activation) with little or none of the side effects associated with conventional anti-inflammatory medicaments. When these compositions are administered in conjunction with conventional anti-inflammatory drugs,

less of the conventional drug is required to achieve the same or similar therapeutic benefit, thereby reducing undesirable side effects associated with the conventional drug.

Example 7 Compositions containing ginkolide, astaxanthin and vitamin C suppress allergen induced asthma

The effect combinations of vitamin C, astaxanthin, and ginkolide on asthma-associated disease parameters ovalbumin(OA) -induced asthma in guinea pigs was evaluated. Twenty-four hours following OA challenge, animals are sacrificed and numbers of inflammatory cells (eosinophils, neutrophils, macrophages) are measured in bronchoalveolar lavage (BAL) fluid; and cAMP and cGMP concentrations in the lung tissue. The experiments were carried out as follows.

Asthmatic model (sensitization procedure):

Male Hartley guinea pigs (250-350 g) were sensitized by intramuscular injections of 0.35 ml of a 5% (W/V) ovalbumin (OA)/saline solution into each thigh on days 1 and 4. Guinea pigs were ready for asthmatic challenge after 25 days of ovalbumin injection. Asthmatic challenge was carried out with ovalbumin aerosol, and bronchoalveolar lavage (BAL) was done 24 hours later. The numbers of eosinophils, neutrophils, and macrophages in the BAL fluid were counted.

Measurement of cAMP and cGMP:

Biopsies from lung tissues, cAMP and cGMP were measured using commercially available radioimmunoassay kits (Amersham). Immediately after sampling, lung biopsies were frozen by means of a Wollenberger clamp prechilled in liquid nitrogen. Samples were powdered with a pestle and mortar in liquid nitrogen and trichloro acetic acid (TCA) was added to the powdered frozen samples (10 ml to every mg of tissue). Samples were further homogenized in the frozen TCA in the braying mortar and then centrifuged at 14,000 x g for 10 min at 4° C. The supernatants were extracted 6 times in water-saturated diethylether, evaporated and assayed for cGMP by radioimmunoassay using liquid scintillation counter (Packard, Tri-Carb 2100TR).

Experimental time course and measurement of cells in BAL fluid:

Twenty-four hours after the aerosol OA challenge, guinea pigs were killed by cervical dislocation and exsanguinated by severing the axillary arteries. Lungs were

lavaged with 50 ml of DulBecco's phosphate-buffered saline (aliquots of 10 ml), which were aspirated after gentle chest massage. BAL fluid was centrifuged at 2200 rpm (1100 x g) for 10 min, supernatant was aspirated, and pellets were resuspended in 5 ml 0.25% NaCl to lyse residual erythrocytes. This dispersion was centrifuged at 2200 rpm (1100 x g) for 10 min, supernatant was aspirated, and pellets were resuspended 5 ml 0.9% NaCl. Total cell counts were done by hemocytometry using trypan blue stain. Slides were prepared on a Shandon Cytospin 2 (Pittsburgh, PA) at 300 rpm for 5 min, fixed and stained. Differential cell counts were done using standard morphologic criteria to classify cells as eosinophils, neutrophils, or macrophages, and the results were expressed in cell numbers.

Dosage effects of vitamin C, AX, and GB administered singularly

The dose-responsive effect of AX, EGb761, and vitamin C administered independently for suppression of asthma-associated parameters in allergen-induced, asthmatic guinea pigs was determined. Ibuprophen (IB) was used as the positive control. The results are shown in Tables 4-7. Concentration of inflammatory cells in bronchoalveolar lavage (BAL) fluid and levels of cAMP and cGMP in lung tissue were measured in guinea pigs 24 hours following challenge with OV. Data is reported as mean ± SD of measurements taken in 6 animals per dosage cohort. *$p<0.05$ compared to corresponding values of OV antigen-challenged, drug-free control group.

As shown in Tables 1-3, these components used separately (10 mg/kg AX, 200 mg/kg of vit C, and 10 mg/kg of GB) failed to protect the OV-induced asthma.

Table 4. Astaxanthin (AX)-induced change in asthma-associated parameters

Cohort size: n = 6	**Control, Asthma-free group**	**Control in OV asthma**	**AX 5mg/kg**	**AX 10mg/kg**	**AX 30mg/kg**	**Ax 100mg/kg**	**Ax 200mg/kg**
Eosinophils (cell x 10^6/animal).	1.4±0.2	5.4±0.8	5.0±0.7	4.6±0.6	3.1±0.5*	2.4±0.6*	2.3±0.5*
Neutrophils (cell x	1.1±0.3	5.8±0.7	5.4±0.8	5.1±0.6	3.3±0.8*	2.4±0.5*	2.0±0.4*

10^5/animal)							
Macrophages (cell x 10^6/animal)	1.7±0.5	9.9±1.1	9.6±1.0	8.0±0.8	3.7 ±0.7*	2.70±0.7*	2.6±0.5*
cAMP pmol (mg protein)$^{-1}$	10.9±0.98	6.7±0.58	6.9±0.5	8.1±0.8	9.6±0.8*	12.6±0.8*	12.1±0.5*
cGMP pmol (mg protein)$^{-1}$	2.6±0.26	1.1±0.17	1.2±0.1	1.4±0.1	1.8±0.3*	2.8±0.3*	2.7±0.2*

Table 5. *Ginkgo biloba* (EGb761)-induced change in asthma-associated parameters

Cohort size: n = 6	Control, Asthma-free group	Control in OVA asthma	EGb761 5mg/kg	EGb761 10mg/kg	EGb761 30mg/kg	EGb761 100mg/kg
Eosinophils (cell x 10^6/animal).	1.4±0.2	5.4±0.8	5.1±0.9	5.0 ±0.8	3.5±0.8*	3.1±0.5*
Neutrophils (cell x 10^5/animal)	1.1±0.3	5.8±0.7	5.6±0.6	5.4±0.6	3.5±0,7*	3.3±0.6*
Macrophages (cell x 10^6/animal)	1.7±0.5	9.9±1.1	9.8±1.1	9.4±1.1	4.9 ±1.5*	4.2±0.6*
cAMP pmol (mg protein)$^{-1}$	10.9±0.98	6.7±0.58	6.9±0.6	8.0±0.9	9.8±0.7*	12.0±1.3*
cGMP pmol (mg protein)$^{-1}$	2.6±0.26	1.1±0.17	1.1±0.2	1.1±0.2	1.5±0.2*	1.8±0.3*

Table 6. Vitamin C-induced change in asthma-associated parameters

Cohort size: n = 6	Control, Asthma-free group	Control in OVA asthma	Vitamin C 50mg/kg	Vitamin C 100mg/kg	Vitamin C 200mg/kg	Vitamin C 400mg/kg
Eosinophils (cell x 10^6/animal).	1.4±0.2	5.4±0.8	5.5±0.5	5.5±0.8	4.8±1.1	3.5±0.9*
Neutrophils (cell x 10^5/animal)	1.1±0.3	5.8±0.7	5.4±0.6	5.6±0.8	5.0±0.8	4.7±0.9
Macrophages (cell x 10^6/animal)	1.7±0.5	9.9±1.1	9.5±0.9	9.8±0.9	9.2 ±0.8	7.2±1.5*
cAMP pmol (mg protein)$^{-1}$	10.9±0.98	6.7±0.58	6.8±0.5	7.1±0.8	7.9±0.9	7.9±0.8

cGMP pmol (mg protein)$^{-1}$	2.6±0.26	1.1±0.17	1.1±0.2	1.0±0.2	1.2±0.2	1.5±0.2*

Table 7. Ibuprophen (IB)-induced change in asthma-associated parameters

Cohort size: n = 6	**Control, Asthma-free group**	**Control in OVA asthma**	**IB 10mg/kg**	**IB 100mg/kg**	**IB 500mg/kg**	**IB 1000mg/kg**
Eosinophils (cell x 10^6/animal).	1.4±0.2	5.4±0.8	5.4±0.8	4.7±0.6	3.0±0.8*	2.9±0.5*
Neutrophils (cell x 10^5/animal)	1.1±0.3	5.8±0.7	5.7±0.7	5.0±0.7	3.1±0.9*	3.1±0.5*
Macrophages (cell x 10^6/animal)	1.7±0.5	9.9±1.1	9.5±0.8	9.0±1.3	4.7±1.3*	4.3±0.9*
cAMP pmol (mg protein)$^{-1}$	10.9±0.98	6.7±0.58	7.1±0.6	7.3±0.9	8.2±0.8*	8.1±0.9*
cGMP pmol (mg protein)$^{-1}$	2.6±0.26	1.1±0.17	1.1±0.2	1.1±0.2	1.4±0.2	1.6±0.2*

Determine the optimal doses of the combination of AX, EGb761, and vitamin C for the prevention of asthma in guinea pigs induced by allergen.

The dose-responsive effect of AX, EGb761, and vitamin C administered in combination for suppression of asthma-associated parameters in allergen-induced, asthmatic guinea pigs was determined. The combination of these three components was given each day for each guinea pig as a "cocktail". The results are shown in Table 8. Concentration of inflammatory cells in bronchoalveolar lavage (BAL) fluid and levels of cAMP and cGMP in lung tissue were measured in guinea pigs 24 hours following challenge with OV. Data is reported as mean ± SD of measurements taken in 6 animals per dosage cohort. *$p<0.05$ compared to corresponding values of OV antigen-challenged, drug-free control group.

The combination of AX (10 mg/kg), vit C (200 mg/kg), and EGb761 (10 mg/kg) produced a significant protection against OA-induced asthma, while these concentrations (using separately of each drug) of AX (10 mg/kg), vit C (200 mg/kg), and GB (10 mg/kg) failed to reduce the severity of asthma. These results demonstrate that combinations of

astaxanthin, *Ginkgo biloba* leaf extract and vitamin C exhibit potent anti-inflammatory potential which is equal or superior to Ibuprophen (IB - Table 7). These formulation are superior to IB as they do not cause the gastrointestinal problems typically associates with IB.

Table 8. Change in asthma-associated parameters induced by formulations composed of astaxanthin, vitamin C and *Ginkgo biloba* leaf extract (EGb761).

Cohort size: n = 6	Control, Asthma-free group	Control in OV asthma	5 mg/kg AX + 50 mg/kg Vit C + 5 mg/kg EGb761	10 mg/kg AX + 200 mg/kg Vit C + 10 mg/kg EGb761	30 mg/kg AX + 400 mg/kg Vit C + 30 mg/kg EGb761
Eosinophils (cell x 10^6/animal).	1.52±0.15	5.63±0.90	5.07±0.63	3.44±0.84*	2.95±0.62*
Neutrophils (cell x 10^5/animal)	1.00±0.31	5.92±0.66	5.27±0.74	3.65±0.65*	2.60±0.69*
Macrophages (cell x 10^6/animal)	1.52±0.40	10.18±0.88	9.40±0.96	5.78±1.18*	2.98±0.71*
cAMP pmol (mg protein)$^{-1}$	10.77±0.74	6.75±0.81	7.17± 0.61	9.28±0.94*	13.00±0.76*
cGMP pmol (mg protein)$^{-1}$	2.72±0.12	1.02±0.15	1.17± 0.19	2.09±0.48*	2.84±0.28*

These data demonstrate the effect of the combination of *Ginkgo biloba* (EGb761) astaxanthin (AX) and vitamin C to suppress features of asthma pathogenesis. Specifically, this formulation suppressed inflammatory processes including decreased inflammatory cell infiltration and cAMP and cGMP concentration in tissue. These data support the usefulness of the formulation described herein to alleviate symptoms of asthma and other inflammatory disorders that are associated with similar inflammatory processes. For example dry eye, an aqueous tear-deficient dry eye syndrome which results disruption of the ocular surface-lacrimal gland homeostatic cycle. Dry eye is characterized by dry inflammation of the lacrimal gland, and presence of a dense infiltrate

of inflammatory cells in and around the tear duct causing high localized expression of pro-inflammatory cytokines.

Example 8 Effect of asataxanthin (ASX), ginkgolide B and their combinations on PHA-mediated induction of CD3+CD25+ or CD3+HLA-DR+ lymphocytes in human peripheral blood mononuclear cells (PBMC).

Cells from 3-12 asthma patients were cultured 24 hours with 50 μg/ml PHA. PBMC evaluated by 2-color flow cytometry were gated for CD3+ and analyzed for CD3+CD25+ or CD3+HLA-DR+ as a percentage of the CD3+ population. Results are reported as stimulation indices (SI) calculated as the ratio of %CD3+CD25+ cells or % CD3+HLA-DR+ cells in fully-stimulated cultures to %CD3+CD25+ or %CD3+HLA-DR+ respectively in cells treated with PHA plus ASX, GB, or combinations thereof. Bolded entry demonstrates effect of GB + ASX, more effective than either at same concentration in culture. The results are shown in Table 9 below.

Table 9

Culture condition	CD3+CD25+ Stimulation Index	P versus Stimulated cultures	N Subjects	CD3+HLA-DR+ Stimulation Index	P versus Stimulated cultures	N Subjects
Unstim	0.05± 0.01	0.000	12	0.40 ± 0.08	0.008	4
Stim	1.0 ± 0.00		11	1.00 ± 0.00		4
ASX 10^{-8}M	0.95 ± 0.05	0.174	8	0.68 ± 0.16	0.067	4
ASX 10^{-7}M	0.90 ± 0.05	0.032	10	0.71 ± 0.23	0.164	3
ASX 10^{-6}M	0.96 ± 0.10	0.350	10	0.83 ± 0.23	0.267	3
ASX 10^{-5}M	0.95 ± 0.06	0.205	11	0.79 ± 0.19	0.193	3
ASX 10^{-4}M	0.49± 0.09	0.000	11	0.85 ± 0.18	0.191	3
GB 10^{-8}M	0.90 ± 0.07	0.052	9	0.73 ± 0.12	0.077	3
GB 10^{-7}M	0.92 ± 0.08	0.161	10	0.78 ± 0.20	0.194	3
GB 10^{-6}M	0.83 ± 1.0	0.060	10	0.66 ± 0.06	0.001	3
GB 10^{-8}M + ASX 10^{-8}M	0.90 ± 0.06	0.057	7	0.74 ± 0.15	0.116	3
GB 10^{-8}M + ASX 10^{-7}M	0.90 ± 0.05	0.056	8	0.91 ± 0.17	0.317	4
GB 10^{-8}M + ASX 10^{-6}M	0.90 ± 0.06	0.072	9	0.79 ± 1.0	0.055	4
GB 10^{-7}M + ASX 10^{-8}M	0.82± 0.04	0.004	6	0.82 ± 0.11	0.071	4
GB 10^{-7}M + ASX 10^{-7}M	**0.88 ± 0.05**	**0.018**	**10**	0.78 ± 0.09	0.048	4
GB 10^{-7}M +						

ASX 10^{-6}M	0.94 ± 0.06	0.188	10	**0.61 ± 0.05**	**0.002**	**4**
GB 10^{-6}M + ASX 10^{-8}M	0.90 ± 0.04	0.031	6	0.81 ± 0.13	0.118	4
GB 10^{-6}M + ASX 10^{-7}M	0.87 ± 0.08	0.074	8	0.73 ± 0.16	0.094	4
GB 10^{-6}M + ASX 10^{-6}M	0.93 ± 0.07	0.185	7	0.80 ± 0.17	0.174	3

Not shown on table: **ASX 10^{-8} M versus GB 1010^{-7} M + ASX 10^{-8}M p= 0.041 for CD25**

Other embodiments are within the following claims.